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Hypermethylation of *GSTP1*, *CD44*, and E-Cadherin Genes in Prostate Cancer Among US Blacks and Whites

Karen Woodson,[1]* Richard Hayes,[3] Louise Wideroff,[2] Liza Villaruz,[1] and Joseph Tangrea[1]

[1]*Cancer Prevention Studies Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland*
[2]*Applied Research Branch, Division of Cancer Control and Population Sciences, National Cancer Institute, Bethesda, Maryland*
[3]*Division of Epidemiology & Genetics, National Cancer Institute, Bethesda, Maryland*

BACKGROUND. In the US, the incidence and mortality of prostate cancer is about twofold higher among US Blacks compared to Whites, suggesting racial differences in prostate tumor occurrence and aggressiveness. The reason for these racial differences is unknown. Epigenetic events such as promoter-region gene hypermethylation may be influenced by environmental exposures and have been implicated in prostate carcinogenesis (by the silencing of tumor suppressors and other regulatory genes).
METHODS. Using real-time methylation-sensitive PCR, we assessed differences in DNA hypermethylation of *GSTP1*, *CD44*, and E-cadherin (three genes thought to be important in the progression of prostate cancer) in archival tumor tissue of black (n = 47) and white men (n = 64).
RESULTS. We found a high prevalence of GSTP1 hypermethylation overall (84%) but no differences by race (89 and 83% in black vs. white men, respectively), tumor stage, or grade. Although CD44 hypermethylation was less prevalent overall (found in 32% of tumors), we observed a 1.7-fold higher frequency among black men (43 vs. 25% in black vs. white men, $P = 0.05$) and a correlation with tumor grade (CD44 was hypermethylated in 10, 42, and 52% of well, moderate, and poorly differentiated tumors, respectively, $P = 0.003$) but not disease stage. The E-cadherin gene was not hypermethylated in any of the tumors. In summary, of the three genes examined, only CD44 hypermethylation differed by race and correlated with tumor grade, independent of race.
CONCLUSIONS. These preliminary findings suggest that differences in gene promoter hypermethylation may potentially underlie racial differences in prostate cancer pathogenesis and should be explored in larger studies. *Prostate 55: 199–205, 2003.*

KEY WORDS: **hypermethylation; GSTP1; CD44; E-cadherin; real-time methylation-sensitive PCR; prostate cancer**

INTRODUCTION

Prostate cancer is the most common cancer and a leading cause of cancer mortality in American men [1]. The incidence and mortality for prostate cancer is about twofold higher in US Blacks than in Whites, with US Blacks experiencing among the highest rates worldwide [2]. Further, US Blacks are more likely to develop

Abbreviation: MSP, methylation-sensitive PCR.

*Correspondence to: Karen Woodson, Cancer Prevention Studies Branch, Center for Cancer Research, National Cancer Institute, 6116 Executive Blvd, MSC 8314, Bethesda, MD 20892.
E-mail: kw114v@nih.gov
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prostate cancer at an earlier age, present with a higher stage, and have a higher rate of metastasis and poorer survival than Whites [3–5]. These racial differences in the incidence and clinical behavior of prostate cancer have been hypothesized to result from differences in genetics, diet, socioeconomic status, lifestyle, and access to medical care [6–8]. Molecular mechanisms underlying racial differences in prostate tumors have not been well-characterized, however.

DNA hypermethylation refers to a type of methylation aberrancy often found in neoplastic cells, in which promoter regions of genes that are normally unmethylated become methylated by the covalent binding of a methyl group to the 5′-cytosine of the dinucleotide pair, CpG (reviewed in Ref. [9]). Methylation of these normally unmethylated promoter regions is thought to contribute to carcinogenesis by silencing expression of tumor suppressor and other regulatory genes. DNA hypermethylation has been shown to silence individual genes considered important in the different stages of prostate cancer progression. For example, inactivation of GSTP1 has been shown to occur early in high grade intraepithelial neoplasia (HGPIN), a prostate cancer precursor lesion [10], whereas E-cadherin and CD44 inactivation occurs later and may be associated with the progression of localized cancer to metastatic disease [11–14]. Hypermethylation may play a role in the inactivation of these genes in prostate carcinogenesis.

Environmental exposures such as diet, hormones, arsenic, and selenium have been shown to affect DNA methylation in experimental models [15–18]. There may also be genetic influences in the susceptibility to aberrant DNA methylation. Here, we studied whether there are differences in DNA methylation of three genes, *GSTP1*, *CD44*, and E-cadherin in prostate cancers from US Blacks and Whites in order to try to elucidate potential molecular mechanisms that may contribute to the increased risk of prostate cancer among black men.

SUBJECTS AND METHODS

HumanTissue Specimens and Cell Lines

Formalin-fixed paraffin embedded prostate tumor tissue was obtained from needle biopsies, transurethral prostatic resections (TURPs), and radical prostatectomy specimens from participants of the Population Health Study (PHS). The PHS was an NIH-sponsored case-control study of prostate cancer among US Blacks and Whites that was conducted in the geographical areas covered by the population-based cancer registry of the Georgia Center for Cancer Statistics, the Metropolitan Detroit Cancer Surveillance System, and the New Jersey State Cancer Registry (ten counties) between 1986 and 1989. The study received Institutional Review Board approval [19]. Tumor tissue was obtained from 144 of the 387 New Jersey cancer cases. H&E stained sections were histologically examined for the presence of tumor cells. Only sections that showed at least 20% of tumor on the slide were used for subsequent DNA extraction ($n = 111$). Core-needle biopsy samples were used in those cases where radical prostatectomy samples were not available ($n = 18$). Men were clinically staged as having localized versus extracapsular disease. Tumor grade was diagnosed prior to common use of the Gleason system. Therefore, tumor grade was categorized as well (roughly equivalent to Gleason <5), moderate (roughly equivalent to Gleason 5–7), and poorly differentiated (roughly equivalent to Gleason 8–10). DNA from three prostate cancer cell lines (DU145, PC3, and LNCAP) was used to validate methylation assays.

Bisulfite Modification

Gene-specific hypermethylation status was determined using real-time methylation PCR (MSP) based on the Taqman Chemistry (Applied Biosystem, Foster City, CA). In this technique, methylated sequences are chemically modified and detected by PCR amplification and hybridization with fluorescent labeled probes. In the modification, bisulfite cleaves off the amino group of the cytosine converting it to a thymine while the 5-m-C which is protected and remains a cytosine. In the PCR step, the methylated sequences are distinguished with specific PCR primers and/or hybridization probes that anneal to CpG sites in the region of interest.

DNA was extracted from one 20-micron tissue section using the PUREGENE DNA Isolation kit (Gentra Systems, Minneapolis, MN) following the manufacturer's instructions. Isolated DNA from one-third of the sample or between 10 and 500 ng of DNA was bisulfite modified following the procedures described by Herman et al. [20] with some modifications. Briefly, 1 μg salmon sperm DNA was added to all samples followed by incubation in 0.3 M NaOH for 15 min at 50°C, mixed with 100 μL of a 5 M bisulfite solution (2.5 M sodium metabisulphite, Merck; 125 mM hydroquinone, Sigma, St. Louis, MO, pH 5.0), and incubated overnight at 50°C, under the exclusion of light. The bisulfite was then removed using the Promega Wizard DNA Cleanup System (Promega Corp., Madison, WI). The reactions were then desulphonated by addition of 3 M NaOH to a final concentration of 0.3 M NaOH followed by ethanol precipitation. The samples were then resuspended in 10 μL H_2O and 1 μL used for subsequent Taqman reactions.

DNA Hypermethylation Assays

Determination of hypermethylation was conducted by real-time methylation sensitive-PCR using the ABI 7900 (Applied Biosystems). Primers and hybridization probes were designed to bind specifically to bisulfite converted methylated sequences in the CpG islands in the promoter of *GSTP1*, E-cadherin, and *CD44* gene (each assay evaluated from four to six individual CpG sites). Optimal primer and probe sets were selected for with the Primer Express software package under conditions specified by Applied Biosystems. The amount of input DNA in each sample was standardized by including internal reference primers that anneal with bisulfite-converted β-actin sequences in the region of the gene with no CpG sites. The primers and probe for each gene were synthesized by Applied Biosystems and are listed in Table I.

The real-time PCRs were carried out in a reaction volume of 15 μl using Taqman Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). Each PCR reaction mixture contained 300 nM of each primer, 100 nM probe, and 1× Taqman Buffer. Amplification and detection were carried out using the following profile: one step at 50°C for 2 min, one step at 95°C for 10 min, and 50 cycles at 95°C for 15 sec and 60°C for 1 min. The sensitivity and specificity of the assays were tested by running standards of serial dilutions with known amounts of methylated DNA (from 50 to 0.05 ng). For GSTP1 and CD44 methylation assays each plate consisted of serial dilutions of methylation-positive prostate cancer cell line (LNCAP) and methylation-negative prostate cancer cell line DU145. For E-cadherin the sensitivity and specificity were determined using human placental DNA treated with *SSI* methyl/transferase according to the manufacturer's protocol (New England Biolabs, Beverly, MA). All data presented were within this range of amplification and all of the assays were performed at least twice. The precise quantitation of hypermethylated DNA was determined by reading the midpoint of the linear portion of the S-shaped real-time curves, called the Ct point or threshold cycle. The Ct refers to the number of cycles it takes a sample to reach a specific fluorescence threshold. Samples with Ct below 50 were considered to have promoter-region hypermethylation.

Statistical Analyses

Analyses were carried out using the STATA software package (College Station, TX). Differences in patient or tumor characteristics by race were examined using Student's *t*-test for continuous variables and the chi-square test for categorical variables. Differences in the proportion of patients' tumor sample DNA exhibiting GSTP1 or CD44 hypermethylation by race,

TABLE I. PCR Primers/Hybridization Probes Used for Real-Time *MSP* Gene-Specific DNA Hypermethylation Assays

Gene	PCR forward	PCR reverse	Fluorescent hybridization probe
GSTP1	5′-AGAGGGAAAGGTTTTTTCGGTT-3′	5′-GCGAACTCCCGCCGA-3′	6FAM-5′-TGCGCGGCGATTTCGGG-3′TAMRA
CD44	5′-TGTCGTTGAGTTTGGCGTCGTAGA-3′	5′-CCGCACCCATCTTACTACCC-3′	6FAM-5′-CCTACGACGAACACTCA-3′MGBNFQ
E-cadherin	5′-GAATTAGAATCGTGTAGGTTTTATAATT-TATTTAGA-3′	5′-CCGACCACAACCAATCAACA-3′	FAM-5′-ACCTCGCATAAACGCGATA-3′MGBNFQ
B-actin	5′-GGTGGAGGTAGTTAGGGTTATTTGTA-3′	5′-CCACAAAATCACACTTAACCTCATTT-3′	FAM-5′-CACTTTTATTCAACTAATCTC-3′MGBNFQ

FAM, reporter dye; TAMRA-quencher dye; MGBNFQ, molecular-groove binding non-fluorescence quencher hybridization probes which allow for using probes with a lower melting temperature as needed for AT-rich sequences (Applied Biosystems, Foster City, CA).

tumor grade, or disease stage were examined using the Fishers Exact or the chi-square test. The associations between gene promoter hypermethylation and race, disease stage, and tumor grade were estimated using logistic regression (odds ratio, OR, and 95% confidence intervals, CI). Two-sided statistical tests were used throughout; *P* values equal or less than 0.05 were considered significant.

RESULTS

The patient and tumor characteristics for the prostate cancer cases according to race are presented in Table II. Forty-two percent of the cases were Black and the overall mean age was 67 years. Nearly 80% of the cases had clinically localized disease and 60% had well to moderately differentiated tumors. There were essentially no differences in age of diagnosis, tumor stage or grade, or family history between black and white men.

We first tested our DNA hypermethylation assays of GSTP1, CD44, and E-cadherin using prostate cancer cell lines with known methylation status (GSTP1 and CD44) or artificially methylated human placental DNA (E-cadherin gene). Consistent with prior reports [21–23], our assay detected GSTP1 and CD44 hypermethylation in LNCAP cells but not in DU145 or PC-3 cells. Since E-cadherin was not hypermethylated in any of the cell lines, we used artificially methylated human placental DNA for the E-cadherin methylation assay development. A representative amplification plot of the real-time MSP of GSTP1 from serial dilutions of the methylation-positive prostate cancer cell line (LNCAP) and prostate tumor specimens is shown in Figure 1. The assay was in linear range starting from 50 ng of LNCAP DNA down to 1:10,000 dilution or 50 pg of target DNA. The assay was highly specific for GSTP1 methylated sequences as 50 ng of DU145 cells showed no amplification. The amplification plots of CD44 and E-cadherin were similar indicating our CD44 and E-cadherin hypermethylation assays are also highly sensitive and specific (data not shown).

Consistent with previous studies [10,21,24], GSTP1 hypermethylation was highly prevalent in our tumor specimens (Table III). We observed GSTP1 hypermethylation among 84% of all patients, with no difference in the frequency between black and white cases (83% among Blacks vs. 87% among Whites, $P = 0.64$). CD44 hypermethylation was less prevalent, found in only 32% of total cases. There did appear to be statistically significant racial differences in CD44 hypermethylation; the prevalence was about twofold higher among black men (Table III). GSTP1 and CD44 methylation was tumor-specific; we did not observe methylation of these genes in any of the adjacent normal prostatic tissue from the radical prostatectomy specimens tested ($n \sim 15$). There was no difference in the prevalence of hypermethylation for either gene among DNA extracted from biopsy, TURP, or radical prostatectomy sample.

Since DNA hypermethylation of some genes has been shown to be associated with aggressive disease characteristics in other cancers and in other prostate cancer studies [25,26], we were interested in whether hypermethylation status correlated with disease stage or grade in our population. We were particularly interested in CD44 since it is involved in cell-adhesion and loss of its protein expression level has been shown to be associated with higher Gleason grade in clinically localized prostate cancer [13]. In our population, CD44 hypermethylation was correlated with tumor grade but not with stage of disease. There were no differences in GSTP1 hypermethylation according to stage (in 84% of regional/distant metastatic cancer compared to 86% in situ or local tumors) (Table IV).

TABLE II. Patient and Tumor Characteristics According to Race, US Men, Population Health Study

	All cases n = 111 no. (%)	Black men n = 47 no. (%)	White men n = 64 no. (%)	*P*-value*
Age (years), mean (SD)	66.9 (8.2)	67.9 (7.2)	66.2 (8.8)	0.30
Family history	7 (6.3)	3 (6.4)	4 (6.3)	0.69
Stage[a]				
Localized disease	84 (79.2)	36 (80.0)	48 (78.7)	0.87
Extracapsular	22 (20.8)	9 (20.0)	13 (21.3)	
Grade, differentiated[b]				
Well	29 (30.9)	12 (27.9)	17 (33.3)	0.85
Moderate	38 (40.4)	18 (41.9)	20 (39.2)	
Poor	27 (28.7)	13 (30.2)	14 (27.5)	

*Two-side *P*-value based on Student's *t*-test (age and chi-square).
[a]Information on state of disease unavailable for five cases.
[b]Information on tumor grade unavailable for 17 cases (4 (9%) black men and 13 (20%) white men).

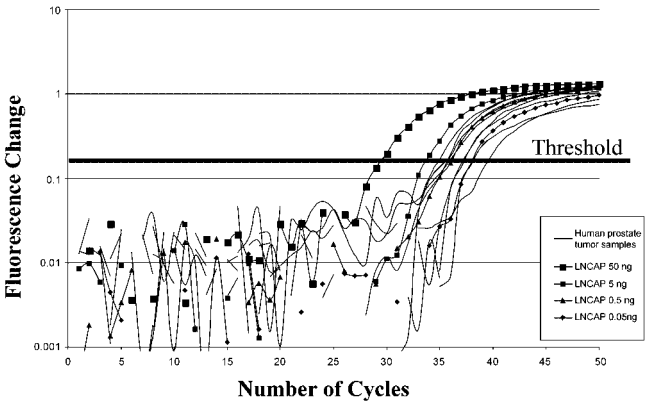

Fig. 1. Representative real-time MSP amplification plots of serial dilutions from LNCAP prostate cancer cells and human prostate tumor specimens. The assay was in linear range from 50 ng down to 1:10,000 or 50 pg of target DNA. The x-axis indicates the number of PCR cycles. The y-axis indicates the change in fluorescence signal normalized to a passive reference signal (log scale).

In logistic regression models we estimated the association (OR) between CD44 hypermethylation and race. Black men were twice as likely as white men to exhibit CD44 hypermethylation (OR = 2.2, 95% CI 1.0–4.9). This association persisted when we controlled for tumor grade (OR = 1.7, 95% CI = 0.7–4.2).

CD44 hypermethylation was only observed among those tumor specimens that had GSTP1 hypermethylation. Among all tumor specimens, 16% (18 of 111) had neither gene hypermethylated, 51% (57 of 111) had GSTP1 but not CD44 hypermethylation, and 32% (36 of 111) had hypermethylation of both genes.

DISCUSSION

In this study, we evaluated differences in promoter-region hypermethylation of three genes in order to elucidate potential molecular mechanisms explaining the racial differences in the incidence and clinical behavior of prostate cancer in the US. We found no racial differences in the prevalence of GSTP1 hypermethylation, a gene thought to be inactivated early in prostate cancer (observed in 70% of PIN lesions) [10]. Interestingly, hypermethylation of CD44, a gene involved in cell adhesion and cell–cell interactions and correlated with the metastatic potential of tumors was significantly increased in black men. Further, CD44 hypermethylation was highly correlated with tumor grade, with 52% of high-grade or poorly differentiated tumors having CD44 hypermethylation compared to it being found in only 10% of well-differentiated tumors. E-cadherin was not hypermethylated in any of the tumor samples examined.

The high prevalence of GSTP1 hypermethylation in our study population is consistent with previous reports demonstrating between 75 and 94% of GSTP1 hypermethylation among prostate cancer tumors [10,21,24]. GSTP1 is a member of a family of enzymes that catalyze the detoxification of electrophillic compounds including a number of xenobiotics and carcinogens, by conjugation to glutathione [27]. Hypermethylation of the CpG island in the promoter of GSTP1 is correlated with loss of gene expression. GSTP1 hypermethylation appears to be specific to cancer and is thought to occur early in carcinogenesis: it is observed in about 90% of cancers and 70% of PIN lesions but not in BPH or normal prostatic tissue [10,21,24]. Our findings suggest that GSTP1 may be useful as an early detection molecular marker but has no prognostic utility.

CD44 is an integral membrane glycogen protein that plays an important role in lymphocyte homing, cell–cell adhesion, and cytoskeletal interactions with the extracellular matrix [28]. Decreased expression of CD44 and its isoforms have been observed in the progression and metastasis of prostate cancer [11–13]. Hypermethylation of the CpG islands in the promoter-region has been associated with decreased CD44 RNA and protein levels [22,23]. Our findings demonstrating CD44 hypermethylation in 32% of tumors overall is lower than previous reports showing CD44 hypermethylation in between 68 and 77% of tumors [29,30]. Differences in assay techniques may account

TABLE III. Gene-Specific DNA Hypermethylation in Prostate Cancers According to Race, US Men, Population Health Study

Gene-specific hypermethylation	All cases n = 111 no. (%)	Black men n = 47 no. (%)	White men n = 64 no. (%)	*P*-value*
GSTP1				
Yes	93 (83.8)	39 (89.3)	54 (83.3)	0.84
No	18 (16.2)	8 (10.7)	10 (16.7)	
CD44				
Yes	36 (32.4)	20 (43.0)	16 (25.0)	0.05
No	75 (67.6)	27 (57.0)	48 (75.0)	

*Two-side *P*-value based on chi-square distribution.

TABLE IV. Gene-Specific DNA Hypermethylation in Prostate Cancers According to Disease Stage and Grade, US Men, Population Health Study

	GSTP1 MS+ (%)	*CD44 MS+* (%)
Stage[a]		
Localized disease (n = 84)	68/84 (81.0)	26/84 (31.0)
Extracapsular (n = 22)	20/22 (90.9)	7/22 (31.8)
	$P = 0.27$*	$P = 0.94$*
Grade, differentiated[b]		
Well (n = 29)	22/29 (75.9)	3/29 (10.3)
Moderate (n = 38)	33/38 (86.8)	16/38 (42.1)
Poor (n = 27)	24/27 (88.9)	14/27 (51.9)
	$P = 0.34$*	$P = 0.003$*

*Two-side *P*-value based on chi-square distribution.
[a]Information on stage of disease unavailable for five cases.
[b]Information on tumor grade unavailable for 17 cases (4 (9%) black men and 13 (20%) white men).

for some of the variability of results. Prior studies used methylation-sensitive restriction fragment PCR techniques. This technique can produce false positives if the DNA is not completely digested by the methylation-sensitive endonuclease. It is unlikely that our assay was not sensitive enough since in this study, CD44 hypermethylation was evaluated using real-time methylation-sensitive PCR techniques based on the Taqman Chemistry, a technique considered to be highly sensitive (we were able to detect down to 50 pg of methylated DNA). Another potential source of variation in the levels of CD44 hypermethylation across studies may be due to population differences such as relative differences in tumor grade or disease stage across studies. Our population consisted largely of men with localized disease (~80%) and if CD44 methylation is a later molecular event in prostate cancer progression, this may explain the lower prevalence we observed.

Our findings of a strong correlation of CD44 hypermethylation with tumor grade indicate that CD44 hypermethylation status may have prognostic implications. Other studies evaluating CD44 hypermethylation in prostate tumors did not report data on tumor grade. Kito et al. [30], however, found a correlation between CD44 hypermethylation and disease stage in a Japanese population (CD44 hypermethylation was observed in 37% of stage B versus 80% stage D tumors). We did not observe a correlation between CD44 hypermethylation and disease stage but our ability to detect differences may have been attenuated by the small proportion of men in our population with extracapsular or advanced disease. Gene-specific hypermethylation has been demonstrated to have clinical implications in cancers at other sites. For example, hypermethylation of CDH1 and FHIT was shown to be associated with significantly poorer survival among bladder cancer patients [26]. In another study, the presence of APC hypermethylation in serum and/or tumor tissue of patients with adenocarcinoma of the esophagus was predictive of poorer disease-specific survival [25].

Of note is the absence of E-cadherin hypermethylation in any of the tumors examined in our study. Although E-cadherin has been shown to be lost in a high proportion of prostate tumors with higher Gleason grade [14], the mechanism by which it is lost is not known. Since data on gene or protein expression of E-cadherin in the tumors from this study is unavailable, we cannot comment on whether E-cadherin is not lost or is lost but not hypermethylated. E-cadherin has been shown to be hypermethylated in tumors at other sites and in DUPro1 prostate cancer cell lines, but hypermethylation of E-cadherin has not been demonstrated in prostate tumors thus far. Future studies correlating E-cadherin hypermethylation and the extent of E-cadherin protein expression in human prostate tumors are being planned.

Racial differences in molecular markers may indicate differing etiologic mechanisms in prostate carcinogenesis between US Blacks and Whites. We were interested in changes in DNA hypermethylation patterns in tumors since this may reflect specific environmental exposures. For example, deficiency of folate and methionine led to DNA hypo- and hypermethylation in lung and liver tumors in rodent models [15]. In vitro studies demonstrated arsenic exposure increases DNA methyltransferase activity and concomitant DNA hypermethylation [16]. Other studies have reported differences in tumor molecular markers between US black and white prostate cancer patients. For example, over-expression of caveolin-1 was found in a higher proportion of tumors from Blacks compared to Whites [32]. This may be an indication of a more aggressive disease phenotype since caveolin-1 overexpression has been associated with the incidence of both recurrent and metastatic prostate cancer [33]. Another study found the apoptotic index (ratio of number apoptotic cells/1,000 tumor cells) for prostate tumors from US Blacks was about threefold higher than Whites [34].

In summary, although GSTP1 hypermethylation was highly prevalent in our study sample, it was not correlated with either race or tumor grade. On the other hand, we observed significant differences in CD44 hypermethylation by both race and grade. In conclusion, these preliminary findings suggest that gene promoter hypermethylation may play a role in racial differences in prostate cancer pathogenesis and should be explored in larger studies.

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